A Study on the Role of Chironomus larvae (Diptera, Chironomidae) as Bioindicator of Lead (Pb) contamination under changing temperature based on its Biochemical assay and Salivary gland chromosomes

SYNOPSIS

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STATE OF THE ART

Aquatic ecosystems are complex networks of biotic and abiotic factors that sustain diverse life forms, including insects. These insects play pivotal roles in ecological processes, acting as primary consumers, decomposers, and prey for higher trophic levels. However, the integrity of aquatic ecosystems is increasingly threatened by anthropogenic activities, leading to the introduction of various pollutants into water bodies. Therefore, disruptions in insect populations due to contaminant exposure can have ripple effects throughout the food web, impacting the abundance and distribution of other aquatic organisms. The recognition of insects as bioindicators of environmental quality reflects the health of aquatic ecosystems through their responses to chemical stressors **(Rosenberg et al., 1993).**

The uptake of pollutants by insects is influenced by a myriad of factors, including the chemical properties of the contaminants, physiological characteristics of the insects, and environmental conditions such as temperature and pH **(Luoma, 1983).** Through experimental studies and modelling approaches, aquatic toxicologists strive to quantify the rates and mechanisms of pollutant uptake by insects, thereby elucidating the dynamics of contaminant bioaccumulation within aquatic food webs **(Rand, 2020).** Climate change induced by temperature variations can profoundly influence the ecology of ectotherms, including their physiological, biochemical and ecological responses **(Calosi et al., 2008).** Temperature variations caused by climate change, especially for thermal extremes, also have profound implications on chemical toxicity to individual organisms, and thus influence the size and structure of their population, the species composition of communities, and the structure and functioning of ecosystems **(Cairns et al., 1975; Cairns et al., 1978; Lau et al., 2014; Zhou et al., 2014).**

Once absorbed, pollutants can exert a multitude of effects on insect physiology, morphology, and behavior. At the cellular level, contaminants may disrupt biochemical pathways, interfere with enzyme function, or induce oxidative stress, leading to cellular damage or death **(Lurling, et al., 2007).**

Metals play crucial biochemical roles in organisms, but they may become carcinogenic and toxic when their concentrations exceed the necessary levels.

Lead is recognized as a serious pollutant based on its persistence, toxicity, bioaccumulation, and extensive use in industry **(Jhonnson & Corelle, 1989; Agg & Zabel, 1990).** Exposure to lead results in several cytogenetical effects on freshwater biota which will adversely affect the 'fitness' of a population, including reducing the rate of cell division **(Muro & Goyer, 1969)** and inducing a range of chromosomal aberrations **(Sharma et al., 1998; Michailova Belcheva, 1990; Lerda, 1992).**

Among biological indicators, Chironomids are distinguished because they make up the most widespread insect family and they have received attention from researchers worldwide due to their outstanding abilities as biological indicators of environmental conditions **(Pinder, 1986)** and reflect early warning signs for detecting toxic pollutants in the environment.

Chironomids belong to the Chironomidae family within the order Diptera. Chironomids are commonly referred to as true flies, non-biting midges, and blind mosquitoes. Chironomids are closely related to mosquitoes (Culicidae) and biting midges (Ceratopogonidae) **(Armitage et al, 1995)**. Chironomids are an important food source for large predatory invertebrates, fishes, and birds **(Hudson et al., 1990).** The occurrence of deformities in the mentum and epipharyngis showed a strong correlation with contaminants, as well as a decrease in pH and dissolved oxygen levels **(Rawal et al., 2019).** These deformities play a direct role in the transformations of contaminants within freshwater habitats. Polytene chromosomes of various Chironomid larvae species exhibit both structural and functional changes in response to heavy metals **(Michailova et al.,2012). Bettinetti (1999), Michailova et al., (1998) and Michailova et al., (2009)** detected inhibitions in the functional activity of key structures, specifically the Balbiani Rings and Nuclear organiser after exposure to toxicants.

Morphological deformities, structural and functional changes in salivary gland polytene chromosomes, and enzymatic activity of Chironomids larvae could be used as a tool for assessing early warning of the potential long-term impacts of heavy metal on aquatic organisms.

PROBLEM TO BE INVESTIGATED

Effect of different concentration of Pb to the Chironomus larvae to ascertain the LC_{50} value with respect to different temperature and the related changes in biochemical assay and salivary gland chromosome of the organism.

OBJECTIVES

The experimental design is laid on to study the metal uptake in Chironomus larvae under different temperature regime and the associated changes on biochemical parameters and salivary gland chromosome. The main aim of the study is as follows:

- Collection and rearing of Chironomus larvae.
- Maintenance of egg mass and larval instars under lab conditions
- Exposing the test animals to heavy metal Lead at different temperature regime of 20,25,30 and 35°C for 24,48 hours
- Testing the physicochemical property of water (pH, DO. TDS, Conductance, and benthic soil carbon content
- Calculation of LC_{50} value to ascertain the sublethal dose of exposure of lead.
- Estimation of lead accumulation in body tissue of the test animal through ICP
- Assessment of total protein and acetylcholinesterase from homogenate of larva
- Preparation of slides to check chromosomal alteration.
- Statistical analysis utilising Probit analysis, Mean, Std Deviation, t test.

HYPOTHESIS

The research proposal is based on the hypothesis that led (Pb) being one of the most potent heavy metal pollutants, has damaging effect on aquatic ecosystem. Chironomus larvae can be used as environmental bioindicator tools attributing to its physiological activity to survive in hypoxic and contaminated environment. The change in its protein content and acetylcholinesterase activity will reveal the mode of distress caused due to polluting effects of lead. Moreover, the study will help to identify any deformity or alteration in salivary gland chromosomes of the Chironomus larvae that will highlight its role as a bioindicator of heavy metal contamination in freshwater ecosystem with change in temperature.

REVIEW OF LITERATURE

Pollution levels have surged significantly since the dawn of the Industrial Revolution in developed nations and the situation continues to deteriorate. Various methods, including physical, chemical, and bioindication approaches, can be utilized to assess the level of pollution.

In recent years there has been increasing interest in employing biological indicators to monitor environmental pollution caused by heavy and toxic metals. The bioindicator approach relies on analysing the heavy metal content of an organism and comparing its concentration with the background metal levels. Scientists have used Chironomus as a model organism for the study to trace the cytogenetic effect of heavy metals on the polytene chromosomes, acetylcholinesterase and protein content.

- **Chironomous as potential bioindicator**

Rawal et al., (2019) worked on the status of Chironomids (Diptera) as potential bioindicators. The study suggests that the morphological deformities in Chironomid larvae work as a tool for indicating environmental health. In Chironomids, polytene chromosome structures like BRs and NOR, could serve as biomarkers to indicate heavy metal pollution. Chironomids exhibit varied responses to stress caused by toxic substances.

Khdre et al., (2023), worked on Chironomus sp. as a bioindicator to estimate the presence of microplastic contamination and its associated heavy metals in the wastewater sediment. Their study was based on the concentration and characteristics of microplastics in sludge, water, Chironomus larva species, and their tubes. They used Chironomus tubes as indicators for freshwater microplastics. This could serve as a warning sign indicating the buildup of microplastics across all compartments of the aquatic environment. This study indicate that microfibers could affect aquatic fauna by transporting various heavy metals.

Michailova Paraskeva et al., (2012 A), investigated the impact of environmental contaminants on genome stability and enzyme activity in *Chironomus riparius* Mg. collected from Bulgarian and Turkish stations over two years, along with laboratory-reared larvae. Sediment analysis revealed elevated levels of heavy metal pollutants compared to reference data. Genome instability, evidenced by structural and functional change in polytene chromosomes, was observed at significantly higher rates in field sites compared to control and laboratory samples. These findings highlight the sensitivity of genome response and biochemical markers as early warning indicators of environmental contamination. The findings of this study support **Steinberg et al., (2008)** hypothesis that response to pollutants involves complex interactions among genes, proteins, and metabolism. The research demonstrates a clear link between chromosome rearrangements, functional changes, enzyme activity, and trace metal pollution.

Chinmoy Ghosh (2016) studied the impact of heavy metal pollutants on the polytene chromosomes of Chironomus larvae. His findings highlight the polytene chromosomes that serve as a reflection of molecular changes occurring at the DNA level, making them effective indicators for detecting genotoxic concentrations of pollutants in aquatic ecosystems.

Michailova et al., (2012 B) studied the genomic alteration in two species of Chironomidae (Diptera) in the Upper Jurassic limestone area of the Ojcow National Park in Poland attributable to natural and anthropogenic factors. They investigated the effect of natural and anthropogenic factors on the sediment in the river Pradnik and the structural and functional alteration in the salivary gland chromosome of two species of Chironomidae. They identified two types of chromosomal rearrangements i.e., inherited and somatic. In functional alteration, they observed changes in the structure of Balbiani Rings (BRs) and Nucleolar Organizer (NORs) and in result they significantly decreased their transcriptional activity. They also observed the changes in the appearance of the telomere region on the chromosome that was considered as very sensitive to the environment and aquatic ecosystems.

- **Effect of lead on Chironomous larvae**

Iron, Copper, and Zinc play important roles in maintaining the organism's structure, but they can become acutely toxic when their concentrations exceed normal levels, as seen in certain genetic disorders **(Liquat Iram et al., 2023)**. Some heavy metals such as Mercury, Arsenic, and Lead, are not important, even in small concentrations, and can become carcinogenic **(Ng JC et al., 2003)**. Lead and Mercury contribute to autoimmune responses, leading to diseases affecting the nervous system, kidney, circulatory system, and causing arthritis **(Barakat, 2011).** Lead can affect an insect's metamorphosis and reduce environmental fitness. **Corbi et al., (2018),** have emphasized the contamination of aquatic ecosystems as a consequence of the utilization of different heavy metal accumulation including lead, cadmium, chromium, zinc, and nickel as fertilizers for agricultural practices that had a major impact on the water supply of surrounding cultivated areas.

Michailova et al., (2002) studied the effect of lead on the salivary gland chromosomes of *Glyptotendipes salinus Michailova* (Chironomidae, Diptera). They investigated the impact of chronic lead exposure on the salivary gland polytene chromosomes of the chironomids Glyptotendipes salinus under laboratory conditions. Lead exposure led to reduced activity in the nucleolar organizer (NOR) and Balbani Rings (BRS), indicating changes in the functional activity of polytene chromosomes. Additionally, there was an increased incidence of asynapsis

in chromosome G and alteration in the centromere and telomere regions. Although they did not report morphological changes in larvae, yet it was observed that the organisms had slow developmental rates. Overall, the study demonstrated that lead exposure activates the chironomid genome, with changes in polytene chromosomes serving as sensitive indicators of environmental stress.

Johan Lidman et al, (2020) worked on the effect of lead (Pb) contamination on aquatic insect's community composition and metamorphosis. They examined the effect of lead (Pb) on insect abundance, metamorphosis, and adult emergence across nine lakes near an abandoned Pb/Zn mine with varying metal concentrations in sediment and water. The finding highlights the importance of considering metamorphosis in toxicity studies, as metal can have complex and sometimes opposing effects.

IMPORTANCE OF THE STUDY

- **National Relevance**

From India, **Sublette and Sublette (1973)** recorded 21 genera of Chironomidae. **Coffman et al. (1988)** reported fifty-five chironomid genera (35 new to India) from south India. Numbers of investigators have added into generic composition of the chironomids fauna of India which is up to seventy-three in number **(Coffman et al., 1988).** High heavy metal concentrations have been reported in river Damodar sediments **(Bhattacharya et al., 2006)** that may lead to the larval mouthpart deformities. However, the relationship between pollution load and extent of deformities are yet to be established and need further investigations. **Podder et al (2013)** also found that heavy metal lead to deformities and stress condition in *Chironomus striatapennis*.

- **International relevance**

Among the heavy metals, Cd, Pb and Hg, are considered as most hazardous water pollutants. Bioaccumulation of these metals in aquatic organisms is dangerous not only for their own survival and biology, but also for humans because of the possible passage of contaminant through the food chain. In stress situation like increasing concentration of heavy metals in aquatic environment the level of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase in Chironomus are decreased and larvae died due to toxicity of the metals. **Michailova et al., (2002)** studied the effect of lead on the salivary gland chromosomes of indicating changes in the functional activity of polytene chromosomes.

SIGNIFICANCE OF THE STUDY: ITS RELEVANCE TO THE PRESENT-DAY PROBLEM AND NEED OF THE SOCIETY

Chironomids have long been used by limnologists and aquatic ecologists as biotic indicators to classify lakes in terms of trophic status and hypolimnetic oxygen concentration. Distribution of chironomid species can be used as water quality indicators, in lake classification **(Nyman, 2007)** and determining environmental conditions of lakes **(Tellioglu et al., 2008 and Warusawithana and Yatigammana, 2007).** The present study will help to study the deformities and enzyme alterations as useful indicators of heavy metal contamination that can be highly detrimental to the health of aquatic ecosystems. Moreover, the study will also try to find out the uptake mechanism of heavy metal (Pb) in relation to change in temperature thereby highlighting the effect of temperature on sustainability of these aquatic organisms.

RESEARCH DESIGN AND METHODOLOGY

- **Selection of specimen animal**

Freshly emerged IV instar larvae of Chironomus will be considered for the study. The larvae will be collected from local sediment and pools of Ranchi and will be identified. The specific species with better count among others will be selected for the experiment.

- **Rearing of Chironomus larvae**

The larvae will be collected from local pools. These will be placed in a glass container, covered with a net to trap emerging adults. The experimental cage shall be kept in coverage of nylon net to prevent entry of exogenous creatures. Polythene trays (38 X 27 X 6 cm) should be used in the experiment containing soil substratum overlaid with water to build up artificial habitat for growing juveniles. The substrata will be prepared with finely crushed autoclaved soil submerged in 2-2.5 cm of distilled water. Water would be changed weekly. A mini aerator would be used for oxygen supply to maintain aeration till the eggs are hatched and juveniles survive. Illuminating arrangements with alternate light and dark phases in a proportion 13:11 hr will be maintained. One 25 W and one 0 W electric bulbs shall be fitted in each culture cage for rendering light and dark phases with the help of timer.

Egg mass will be sorted out and placed in a petridish (15 cm diameter) containing autoclaved soil and pond water with coverage of nylon net. After 2 -3 days the eggs would hatch, and the first instar larvae will be placed in experimental plastic trays. Once the eggs start hatching the tiny first instar would be transferred to experimental tray. Commercial food used for fish aquarium (finely minced), cellulose powder and dried yeast in mixture (3:2:1) dissolved in a proportion of 1 g in 200 ml of distilled water will be supplied to the newly hatched larvae for further growth.

- **Exposure to heavy metal (Pb) under variable temperature range**

Lead will be used in the form of lead nitrate ($PbNO_{3)}$. Chironomus larvae will be kept in batches of ten. Three replicates will be maintained for the experiment. The larvae would be starved during the experimental period and exposed to graded concentrations of lead nitrate by making appropriate dilutions from a stock solution. For determining LC_{50} values, ten larvae will be placed in 100 ml of test solution of each concentration in glass containers of 200 ml capacity. Mortality criterion will be estimated by the larvae's failure to respond to light, probing with the tip of a blunt needle, and will be recorded at 24 and 48 hr at different temperature of 20 ,25,30, and 35°C. Dead organisms will be removed, and test solutions renewed every 24 hr. Each concentration will be tested in 3 replicates. Acute toxicity of $PbNO_3$ will be determined by estimating median lethal concentrations (LC_{50}) with the help of log-probit analysis.

- **Physico chemical analysis of water sample**

pH, DO, Conductance, TDS, and benthic soil organic carbon would be evaluated for the experiment animals with variable temperature range following the APHA method (between 2015 and 2019).

- **Biochemical estimation**

a) Estimation of accumulated Pb: The larvae from each batch after final exposure will be pooled and will be homogenised in 0.1%HNO_3.The samples will be taken for assessment of Pb accumulation through ICP.
b) Total Protein content will be estimated by Lowry et al. (1951) method.

c) Acetylcholinesterase levels will be checked for the test samples by Ellman et al. (1961) protocol.

- **Chromosomal deformities**

Chironomus larvae will be fixed in alcohol: acetic acid (3:1). Fourth instars larvae will be used for study and preparation of salivary gland chromosomes and external morphology of the mouth. Chromosome preparations will be done according to **Michailova (1989)**. Aceto-alcohol will be used as a fixative. It will be prepared by mixing equal volume of glacial acetic acid and absolute ethyl alcohol at the ratio 1:3. Stain preparation would involve 02 grams of Orcein powder in 100 ml of glacial acetic acid.

- **Statistical analysis**

Mean, Std Deviation and Students t test shall be performed to check the statistical significance of the collected data for verification of result (Statgraphic Centurion).

TOOLS TO BE USED

- Plastic trays
- Aerator
- Nylon Net
- Water analysis kit
- Water bath
- Microscope
- Colorimeter/Spectrophotometer
- Glassware

STAGE - WISE PLAN OF WORK AND TARGETS TO BE ACHIEVED

STAGE I:

- Literature survey to determine mode of experimental design.
- Collection and rearing of larvae.
- Study on Dose response to Pb.

STAGE II:

- Fixing LC 50 value of Pb at different temperature
- Estimation of lead accumulated in body tissue through ICP.
- Study of physicochemical properties of water

STAGE III:

- Estimation of protein and Acetylcholinesterase
- Preparation of slide of salivary gland chromosome
- Statistical analysis
- Thesis writing
- Submission of Thesis

CONTRIBUTION TO THE EXISTING KNOWLEDGE

Aquatic toxicology, a multidisciplinary field at the interface of ecology, chemistry, and toxicology, seeks to unravel the impacts of these pollutants on aquatic organisms, with a particular focus on insects. The study of aquatic toxicology in terms of insects necessitates a comprehensive understanding of the mechanisms underlying the interactions between pollutants, insect physiology, and behaviour. The deformities and other biomarkers of stress in the larvae has given better insights to study the mode of distress brought into the body tissue

of insects thereby highlighting their dose response relationship for heavy metal contaminant and changes in its physiology. Aquatic insects stand out as crucial organisms, as they constitute a significant portion of functional feeding groups that contribute to ecosystem dynamics. Furthermore, they have a noteworthy importance in freshwater monitoring, protection, and conservation due to their sensitivity to respond to unfavourable changes in environmental conditions **(Galden et al, 2001)**

FUTURE WORK

Insect populations serve as integral components of aquatic food webs, linking primary producers to higher trophic levels through complex trophic interactions. Future work shall include investigation and identification of other enzymes that act as biomarkers of stress in these organisms. Moreover, more studies should be conducted to study the chromosomal changes that validate the presence of environmental stressors in its body. Genome instability and alterations in enzyme activity emerge as valuable biomarkers for assessing ecological risks in aquatic systems, capable of indicating exposure and effects of contaminants.

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